Assessment of Resistance of *Bacillus horneckiae* Endospores to UV Radiation and Function of their Extraneous Layer in Resistance

Malcolm M. Zachariah[1], Parag Vaishampayan, [2]
Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California, 91109

Spore-forming microbes are highly resistant to various physical and chemical conditions, which include ionizing and UV radiation, desiccation and oxidative stress, and the harsh environment of outer space or planetary surfaces. The spore's resistance might be due to their metabolically dormant state, and/or by the presence of a series of protective structures that encase the interior-most compartment, the core, which houses the spore chromosome. These spores have multiple layers surrounding the cell that are not found in vegetative cells, and some species have an outer layer of proteins and glycoproteins termed the "exosporium" or a fibrous "extraneous layer" (EL). *Bacillus horneckiae* is an EL-producing novel spore-former isolated from a Phoenix spacecraft assembly clean room, and it has previously demonstrated resistance to UV radiation up to 1000 J/m^2. The EL appears to bind *B. horneckiae* spores into large aggregations, or biofilms, and may confer some UV resistance to the spores. Multiple culturing and purification schemes were tried to achieve high purity spores because vegetative cells would skew UV resistance results. An ethanol-based purification scheme produced high purity spores. Selective removal of the EL from spores was attempted with two schemes: a chemical extraction method and physical extraction (sonication). Results from survival rates in the presence and absence of the external layer will provide a new understanding of the role of biofilms and passive resistance that may favor survival of biological systems in aggressive extra-terrestrial environments. The chemical extraction method decreased viable counts of spores and lead to an inconclusive change UV resistance relative to non-extracted spores. The physical extraction method lead to non-aggregated spores and did not alter viability; however, it produced UV resistance profiles similar to non-extracted spores. In addition to the EL-removal study, samples of *B. horneckiae* spores dried on aluminum coupons and exposed to increasing UV (200-400 nm range) levels (0 to 8.0 x 10^5 kJ/m^2) were tested for viability, which indicated that the maximum UV exposure level that still resulted in viable spores was 5.0 x 10^5 kJ/m^2.

[1] USRP Intern, Biotechnology and Planetary Protection Group, Jet Propulsion Laboratory, California Institute of Technology, University of Oklahoma
[2] Research Scientist, Biotechnology and Planetary Protection Group, M/S 89-102, Jet Propulsion Laboratory

Nomenclature

UV = ultraviolet
ASTM = American Society for Testing and Materials
CFU = colony-forming unit
DTT = dithiothreitol
EtOH = ethanol
EL = extraneous layer
g = gravitational acceleration on Earth's surface
HS = heat shock
MSM = modified Schaeffer's medium
PBS = phosphate-buffered saline
PVA = polyvinyl alcohol
SDS = sodium dodecyl sulfate
Tris•HCl = tris(hydroxymethyl)aminomethane hydrochloride
TSA = tryptic soy agar
TSB = tryptic soy broth
UV = ultraviolet

I. Introduction

Species in the bacterial genus *Bacillus* are able react to some environmental stressors such as nutrient scarcity by forming specialized metabolically dormant cells called endospores (henceforth referred to as "spores").[1] Because of their dormancy and specialized structure of concentric shells, spores are able to resist environmental extremes such as heat and ultraviolet (UV) and gamma radiation, making them ubiquitous throughout Earth.[1] Spores are often used as biological indicators of the efficacy of sterilization processes.[2]

The resistance and ubiquity of these spores are of importance to the National Aeronautic and Space Administration (NASA) by possibly contaminating spacecraft and thus extraterrestrial regions, which requires various cleaning and sterilization procedures under the term "planetary protection."[1] Previous studies have demonstrated that multiple *Bacillus* species were a large fraction of the total bacteria isolated in spacecraft-assembly facilities, areas designed as "clean rooms" with controlled and filtered air circulation, low humidity, low nutrient availability, and specialized decontamination procedures.[1,3]

The sporulation process begins after detection of various environmental indicators, including nutrient levels.[4] The vegetative cell builds an separating wall called a septum, similar to that produced in vegetative cell division, dividing the cell into a smaller forespore area, which contains its own chromosome, and larger mother cell area.[4] Eventually the forespore creates two membranes made of peptidoglycan, the polymer found in vegetative cell walls: the original cell wall and a cortex.[4] Outside of the cortex, a thick proteinaceous shell called the coat is formed.[4] In addition to these structures, some *Bacillus* species contain an additional layer surrounding the spore coat, such as the "exosporium," a loosely fitting shell composed of proteins, lipids, and carbohydrates, or a less well-studied "extraneous layer."[5,6]

The present research was part of a long term project termed Biofilm Organisms Surfing Space (BOSS), which was developed to study various microorganisms that form biofilms, aggregates of multiple cells, and their limits of survival in space and extraterrestrial environments. The present research was focused on one species used in the BOSS project, *Bacillus horneckiae*, which forms spores that have an extraneous layer, which appears to aid in their biofilm formation, and have demonstrated resistance to UV radiation of up to 1000 J/m^2.[6] Figure 1 provides various microscopic images of *B. horneckiae*, including phase contrast (light) microscopy images of vegetative and sporulating cells (Fig. 1A), scanning electron microscopy images of spores in a biofilm and the extraneous layer (Fig. 1A, 1B), and transmission electron microscopy images of a single spore, with the extraneous layer pointed out (Fig. 1D).[6] Since the extraneous layer is not found in all *Bacillus* species, it may play a role in *B. horneckiae*'s UV resistance. So, it would be important to develop a method of removing the extraneous layer without significantly affecting the spores' viability to assess any effects on UV resistance.

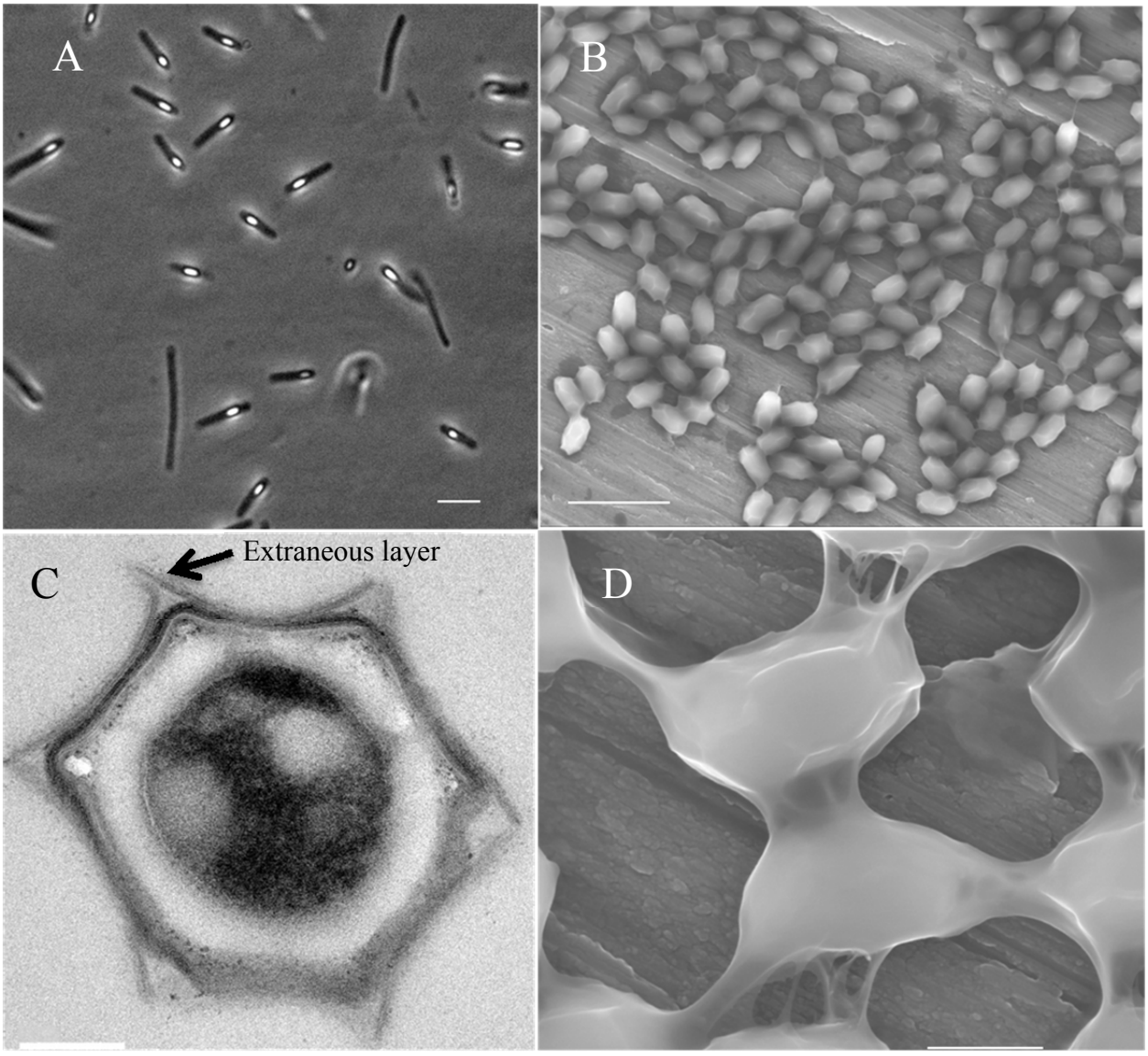

Figure 1. *B. horneckiae* micrographs. *Image A depicts vegetative and sporulating cells under phase contrast (light) microscopy. Images B and D depict scanning electron microscopy of* B. horneckiae *spores in a biofilm, with image D showing the extraneous layer connecting the spores. Image C depicts transmission electron microscopy of a* B. horneckiae *spore, with the extraneous layer pointed out. All images are reprinted with permission from Ref. 6.*

II. Materials and Methods

A. Bacterial Strain and Growth Medium Tests

The strain used in this study, *Bacillus horneckiae*, was isolated from a clean room at the Kennedy Space Center used for assembly of the Phoenix spacecraft.[6] *B. horneckiae* are Gram-stain-positive, rod-shaped vegetative cells that form subterminal (position in the mother cell) oval endospores, which have an extraneous layer.[6]

Culture of *B. horneckiae* vegetative cells was regularly done with either Tryptic Soy Broth (TSB) (14.5 g/L pancreatic digest of casein, 5.0 g/L papaic digest of soybean meal, 5.0 g/L NaCl) or Tryptic Soy Agar (TSA) plates (TSB solidified with 14.0 g/L agar). Sporulation of *B. horneckiae* was induced using a Modified Schaeffer's Medium (MSM), containing 0.1% KCl, 0.012% $MgCl_2$, 0.5 mM $CaCl_2$, 0.01 mM $MnCl_2$, 0.001 mM $FeSO_4$, and 8 g/L Nutrient Broth (Difco). Cultures were inoculated with one isolated *B. horneckiae* colony from a stock TSA culture. All media and subsequent techniques utilized sterile deionized water (dH_2O). This initial culture was itself inoculated from dried spores on an aircraft-grade aluminum coupon that was placed in 100 mL MSM in an incubator-shaker at 32ºC and 120 RPM.

Since the sporulation of *B. horneckiae* takes multiple days for completion, different media and temperature preparations were tested for shorter, while still complete, sporulation times than that of MSM at 32ºC. 1 isolated *B. horneckiae* colony from a TSA plate was inoculated into each of the following media preparations: MSM at 28ºC, MSM with 0.1% glucose at 32ºC, MSM with 1 mM $CaCl_2$ at 32ºC, and MSM with 1 mM $CaCl_2$ + 0.1 mM $MnCl_2$ + 0.1% glucose at 32ºC. The latter medium was based off the "2X SG" medium used in Ref. 7.[7] Phase contrast microscope images using 40X and 100X objective lenses of the cultures (2 µL for each slide) were taken at multiple intervals to monitor the percentage of spores to total cells.

B. Spore Purification Tests

To remove residual vegetative cells from the MSM *B. horneckiae* spore cultures, four purification schemes[8,9] were evaluated. These methods were American Society for Testing and Materials E2111-00 standard (ASTM), ethanol treatment (EtOH), heat-shock (HS), and 3 phosphate buffered saline washes (PBS). A 100 mL MSM culture grown was grown for 7 days at 28ºC and 120 RPM. 2 µL of the original culture were taken for phase contrast microscopy to confirm sporulation. The 100 mL culture was evenly divided into four 50 mL centrifuge tubes. The tubes were then centrifuged at 10,000 x *g* for 15 min at 4ºC. The supernatant was decanted, and each tube was resuspended by vortexing for 20 s into 10 mL of their respective solution: deionized water for the ASTM and HS samples, 50% (v/v) ethanol for the EtOH sample, and PBS for the PBS sample. The EtOH sample was placed in an incubator shaker at 26ºC and 200 RPM for 4 h.

The other three samples were centrifuged at the same settings, their supernatant decanted, and each was resuspended in 10 mL of its respective solutions. The preceding "wash" process (centrifugation, decanting, resuspension) was repeated a third time, after which the ASTM and HS tubes were resuspended in 10 mL of dH_2O, and the PBS tube resuspended in 10 mL PBS. 2 µL of each sample were taken for phase contrast microscopy, and the ASTM and PBS tubes were stored in a 4ºC refrigerator.

The HS tube's contents were placed into a glass test tube and heat shocked in a water bath for 15 min at 80ºC, immediately followed by cooling on ice. The cells were transferred back to a new 50 mL centrifuge tube, washed twice in 10 mL dH_2O, and resuspended in 10 mL dH_2O for storage at 4º C, with 2 µL taken for phase contrast microscopy. The EtOH and HS (transferred back to a 50 mL centrifuge tube) samples were washed twice in 10 mL dH_2O and resuspended in 10 mL dH_2O for storage at 4ºC, with 2 µL taken for microscopy. In addition to microscopy, absence of contamination was determined by quadrant streaking with an inoculating loop (≈10 µL) of each purified spores sample onto a TSA plate, incubating overnight at 32ºC, and observing the morphology of resultant colonies.

C. Extraneous Layer Extractions

Two methods of removing the extraneous layer of *B. horneckiae* were adapted from previous methods used to remove the exosporium of *B. anthracis*: a chemical extraction ("SDS-8 M urea") method and a physical extraction ("sonication") method.[9]

For the chemical extraction method, the concentration of *B. horneckiae* spores purified using the EtOH purification scheme mentioned above were measured using a spectrophotometer. Using water as a blank, the optical density at a wavelength of 600 nm (OD_{600}) of the purified spores was 0.710, and using the metric of an OD_{600} of 0.01 corresponding to 10^6 colony forming units per milliliter (CFU/mL), the purified spores were diluted with dH_2O

to an OD_{600} of 0.5, roughly 5 x 10^7 spores per mL. 1 mL of the OD_{600} = 0.5 spores was placed into a microcentrifuge tube and centrifuged at 10,000 x *g* for 10 min. The supernatant was decanted, and the pellet was resuspended in 1000 µL of an SDS-8M urea sample buffer (50 mM Tris-HCl, pH 10, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 8 M urea, 50 mM dithiothreitol, and 0.02% bromophenol blue). The tube was then placed in a 90ºC water bath to boil for 10 min. The tube was then centrifuged at 10,000 x g for 10 min, the supernatant removed (only 800 µL was stored to avoid taking some of the pelleted cells) into another tube for storage at 4ºC. The pelleted cells were then resuspended in 1000 µL dH_2O, centrifuged at 10,000 x g for 10 min, and resuspended in 1 mL dH_2O for storage at 4ºC. The post-extraction spores were observed with phase contrast microscopy.

Viable counts of the non-extracted and post-extraction spores were taken using serial 1:10 dilutions (in water), followed by spread plating of 100 µL of selected dilutions onto TSA plates, and incubation overnight at 32ºC. The resulting colonies were then counted for each plate, and an original CFU/mL value of each spore solution was calculated using equation (1).

$$\frac{CFU}{mL} = \frac{\#\ of\ colonies}{dilution\ x\ volume\ plated\ (mL)} \quad (1)$$

.

The SDS-8M urea extraction was repeated with the following alterations: 1 mL of purified spores (undiluted) was extracted with 100 µL of the sample buffer. After extraction, the extracted spores and non-extracted spores were each diluted to an OD_{600} of 0.01 to a total volume of 10 mL with dH_2O into a glass petri dish. Using a small stir bar to keep the solution mixed well, the dish cover was removed, and both samples were exposed to 100 µW/cm^2 UV radiation at 254 nm from a low pressure mercury lamp. Each second of exposure corresponded to 1 J/m^2 of irradiation, and at specific times (0, 100, 500, 1000, 1500, 2000 s), an aliquot (200 µL for the 0-500 s exposures and 400 µL for the others) of the spore solutions was removed. For each exposure level, serial dilutions with PBS were done, and two dilutions were used for duplicate spread plates as described above.

For the physical extraction method, 1 mL of EtOH-purified *B. horneckiae* spores diluted 1:10 with Tris-EDTA (TE) buffer were sonicated using a sonicator (Cole=Parmer 4710 series Ultrasonic Homogenizer) with an output control of 4 (power output ~50W). The spores were sonicated in ten 1-minute runs, each separated by 2 minutes of cooling on ice. The spores were then centrifuged as before, and the supernatant was decanted and sterile filtered through a 0.2 µm syringe filter. The spore pellet was then resuspended in 10 mL cold PBS, recentrifuged, its supernatant removed, and resuspended in 10 mL PBS for storage at 4ºC. Subsequently, the spores solution was diluted to an OD_{600} of 0.01 and a volume of 10 mL The sonicated spores, and non-sonicated control, were exposed to UV irradiation as described above with the following alterations: the exposure times were adjusted to 0, 100, 200, 300, 400, and 500 seconds. The aliquots removed were serially diluted and plated for viable counts.

D. UV Exposure Tests

Samples of 10^7 *B. horneckiae* spores on aluminum "coupons" (13 mm diameter) were received for viable count plating. As part of an experimental verification test, these had been exposed to UV irradiation (200-400 nm) using a solar simulator (Argon SOL2000) at an output of 1370 W/m^2 for increasing exposure times that corresponded to the following exposures in kJ/m^2: 1.5 x 10^3, 1.5 x 10^4, 1.5 x 10^5, 5.0 x 10^5, and 8.0 x 10^5. There were three sets of six samples, one for each of the aforementioned exposures and one "dark" 0 kJ/m^2 sample. For each set, a control coupon, which was also unexposed, was also tested. Roughly 50 µL or less of 10% polyvinyl alcohol (PVA) was added to the coupons' surface and allowed to dry for at least 1 hour. The dried PVA film was then peeled, which also removes the spores, and placed into a vial with 2 mL dH_2O to dissolve the spores. The PVA process was repeated a second time to enhance recovery, with the second peeling going into the same vial. 1:10 dilutions of the PVA peeled spore solution was done with PBS, and selected dilutions (chosen from the results of the first set of coupons) were plated onto TSA plates. The plates were placed in an incubator shaker at 32ºC, and colonies were counted after 24 hours. Samples that did not produce any colonies, even at a "dilution" of 10^0, were added (~1.5 mL) to 25 mL molten TSA agar for pour plates, incubated at 32ºC, and observed for any colonies. In addition, the coupons of these 0 CFU samples were placed in 5 mL TSB broth, incubated at 32ºC, and the resulting broth culture was used to inoculate streak TSA plates. From those plates, 5 isolated colonies were removed for frozen cell stocks at -80ºC.

III. Results and Discussion

A. Growth Medium Tests

The MSM culture of *B. horneckiae* spores grown at 28ºC showed 80% spores after 5 days and 95% spores after 6 days, with many spores in small clumps or very large aggregates rather than individually separated spores. The MSM + glucose culture grown at 32ºC at 4 days had >99% vegetative cells, often very long, after 4 days and 90% vegetative cells after 7 days. The MSM + 1 mM $CaCl_2$ culture grown at 32ºC showed 20% sporulation after 3 days and 90% spores after 6 days. The MSM + 1 mM $CaCl_2$ + 0.1 mM $MnCl_2$ + glucose culture grown at 32ºC showed 99% vegetative cells after 3 days and 50% spores after 5 days.

While glucose may increase the total number of cells, its inhibition of rapid and extensive sporulation outweighs these yield benefits. In cultures that had large numbers of vegetative cells, it was difficult to find the percentage of spores because the spore aggregates were less evenly separated than the vegetative cells. With this data indicating no significant timesaving benefits of changing the medium composition, all subsequent sporulation cultures were made with the initial MSM composition and grown at 28ºC and 120 RPM.

B. Spore Purification Tests

Figures 2 and 3 depict the phase contrast microscope images of the four purification schemes. The ASTM, HS, and PBS washes showed motile vegetative cells (an indication of living, not dead, cells) in addition to spores, each around 90-95% spores, a purity similar to that of the spore culture in MSM. The EtOH treated spores microscope images showed close to 99% spores with almost no vegetative cells visible in the portion used for microscopy. The percent of spores to total cells (including vegetative cells) appeared similar to the pre-treatment culture. All TSA plates showed only the colony morphology of *B. horneckiae*, indicating both the vegetative cells and spores were the same. The EtOH purification scheme was chosen for all subsequent purification of spore cultures.

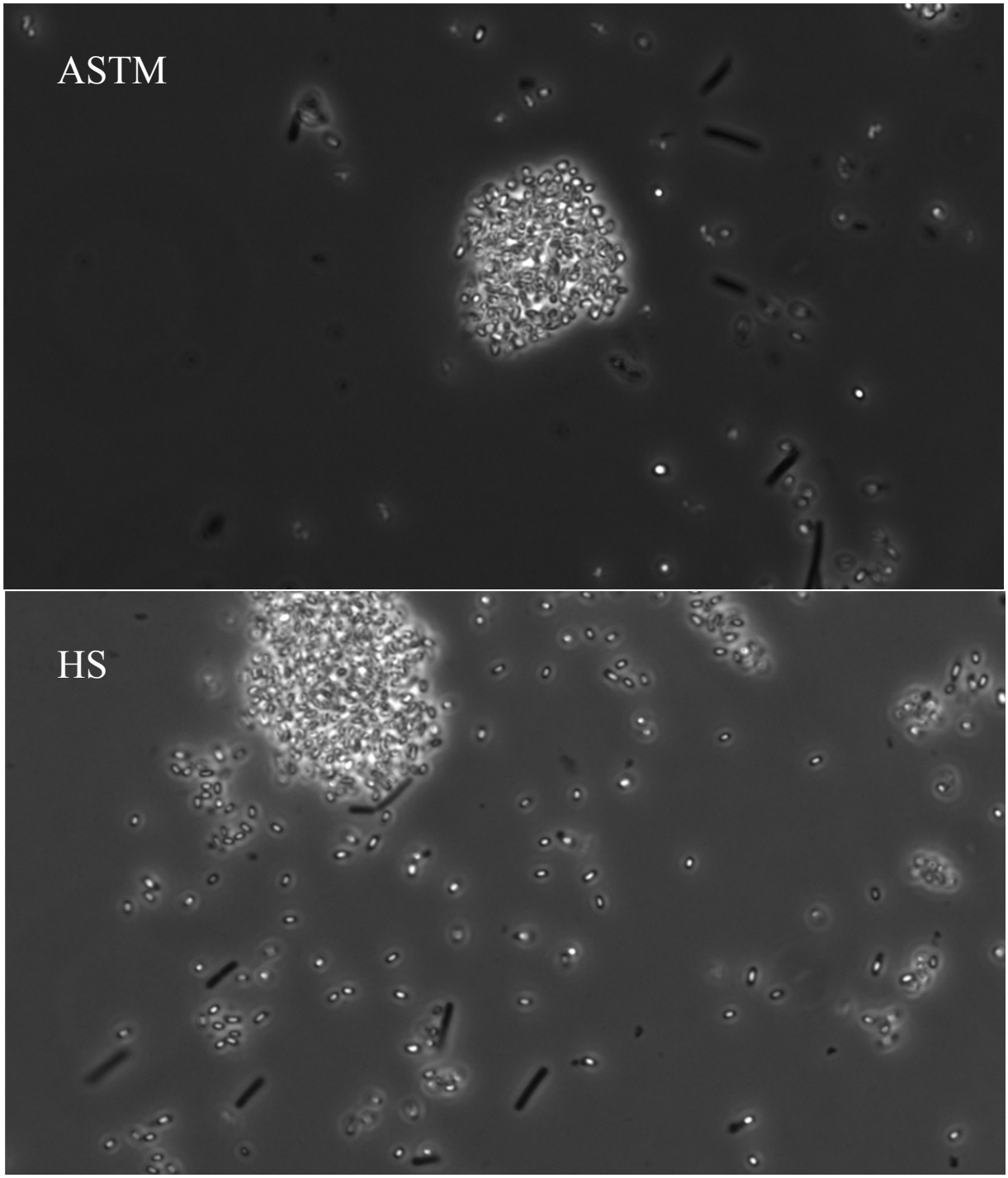

Figure 2. Phase contrast microscope images (100X objective lens) of ASTM (3 H_2O washes) and HS (heat-shock) purified *B. horneckiae* spores.

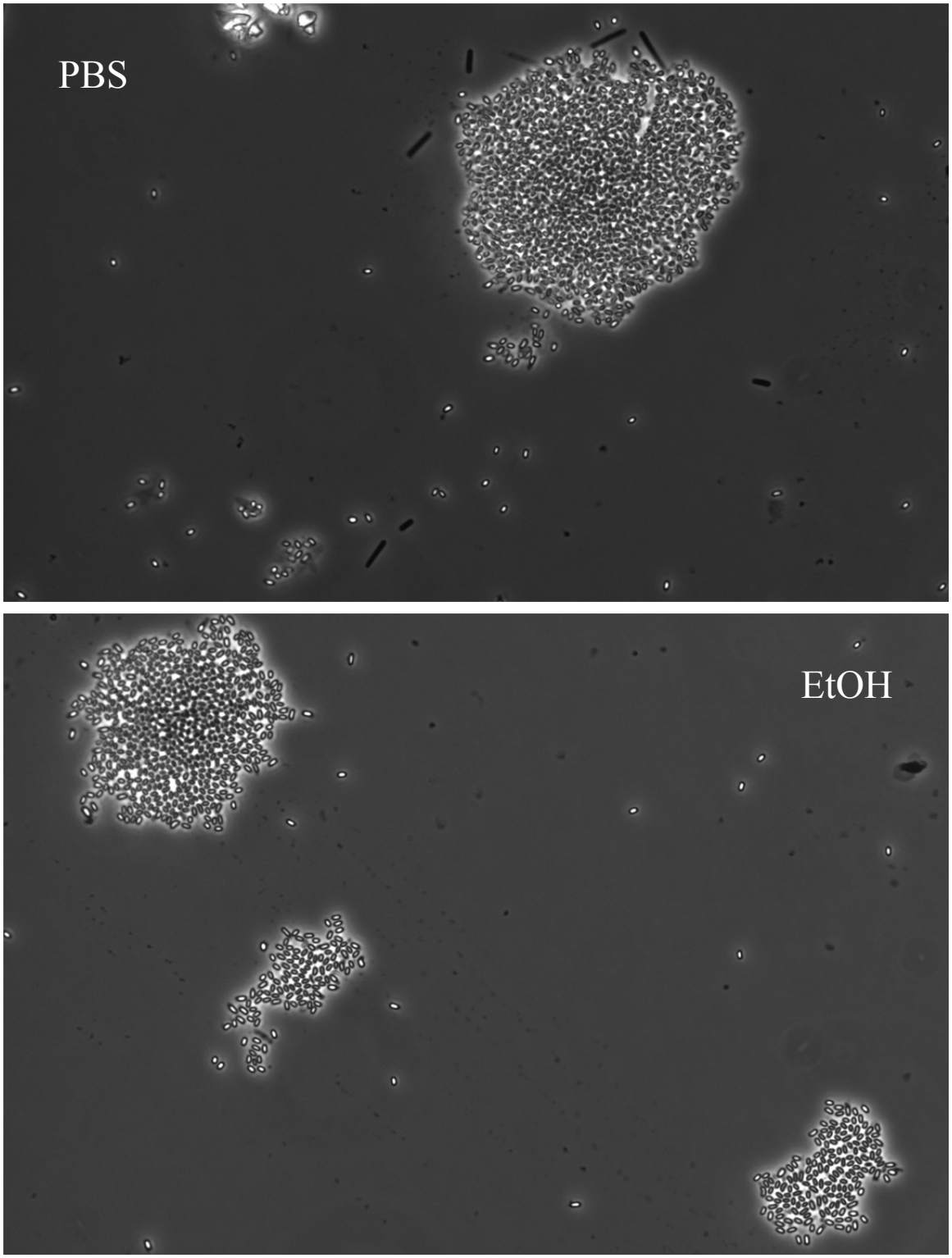

Figure 3. Phase contrast microscope images (100X objective lens) of PBS (3 PBS washes) and EtOH (ethanol-treated) purified *B. horneckiae* spores.

C. Extraneous Layer Extractions

The effect on viability of the initial chemically extracted *B. horneckiae* spores indicated a decrease from 1.5 x 10^8 CFU/mL of pre-extraction spores to 7.5 x 10^5 CFU/mL post-extraction. It is difficult to tell what may have caused the drop in viability, but it is possible a component of the SDS-8M urea sample buffer either inactivates the spores or prevents their germination. When the chemical extraction was repeated and the spores were exposed to UV radiation at 254 nm, the resultant data (not shown) was inconclusive due to the spores diluted to OD_{600} of 0.01 did not produce colonies when undiluted and unexposed (0 J/m^3 exposure level). The large decrease in viable counts also presents a problem if one wants to compare the resistance of the extracted spores to non-extracted spores because the extraction process may have been more complete for spores that died than those that were still viable.

The physical extraction method (sonication) of spores produced a large number of free spores, with very few clumps. Another interesting feature post-sonication was that the spore pellet was unstable in PBS after centrifugation and never completely settled while being stored at 4ºC. These may be indications that the extraneous layer, which fosters aggregation, may have been damaged or removed. Figure 4 depicts the phase contrast microscope images of the sonicated spores. The UV experiment of the sonicated spores (with a non-sonicated control) showed similar results, with both spore solutions decreasing from around 10^6 CFU/mL at 0 J/m^2 to 10^2 CFU/mL at 500 J/m^2. Figure 5 depicts the results of that UV experiment.

In the future, scanning and tunneling electron microscope images will be taken to conclusively determine if the extraneous layer was actually removed (and the extent of removal) by both purification processes.

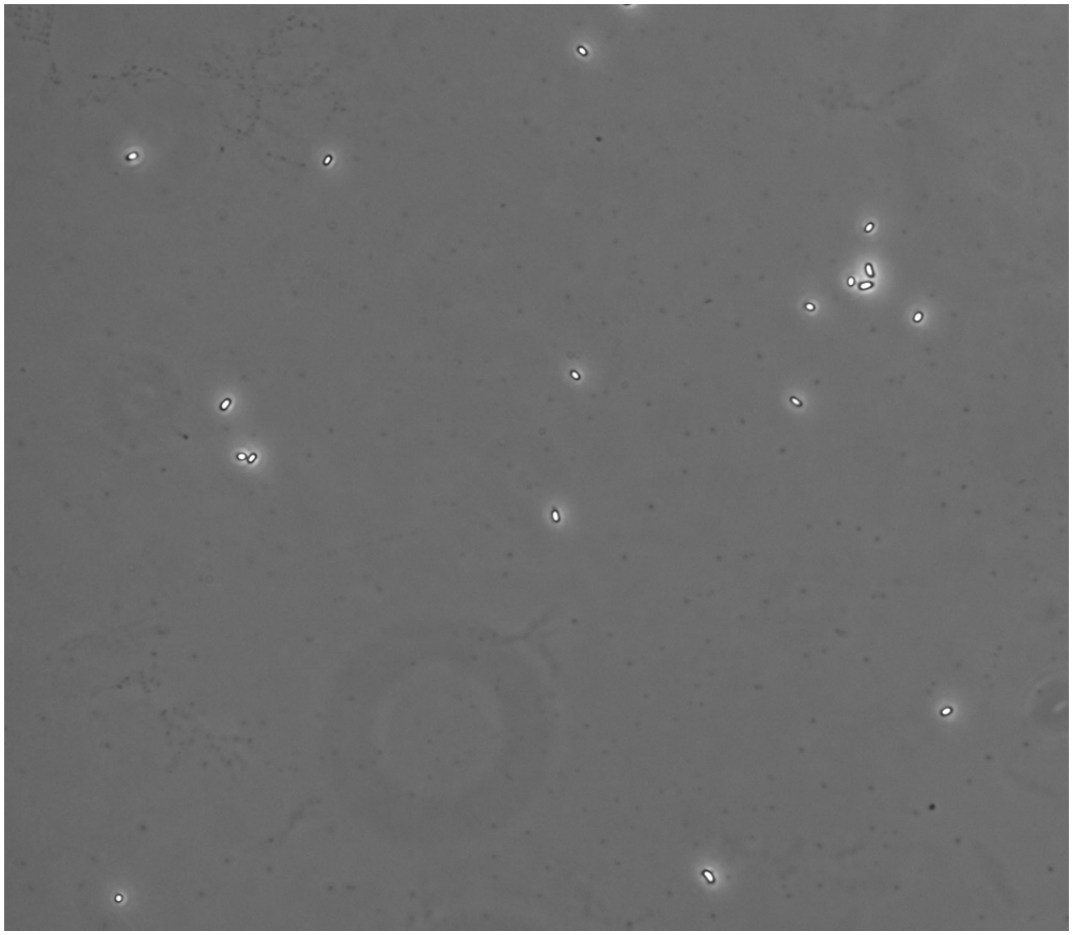

Figure 4. Phase contrast microscope image (100 X objective lens) of physically extracted (sonicated) *B. horneckiae* spores. *Unlike the pre-extracted spores, the sonicated spores exhibited limited aggregation.*

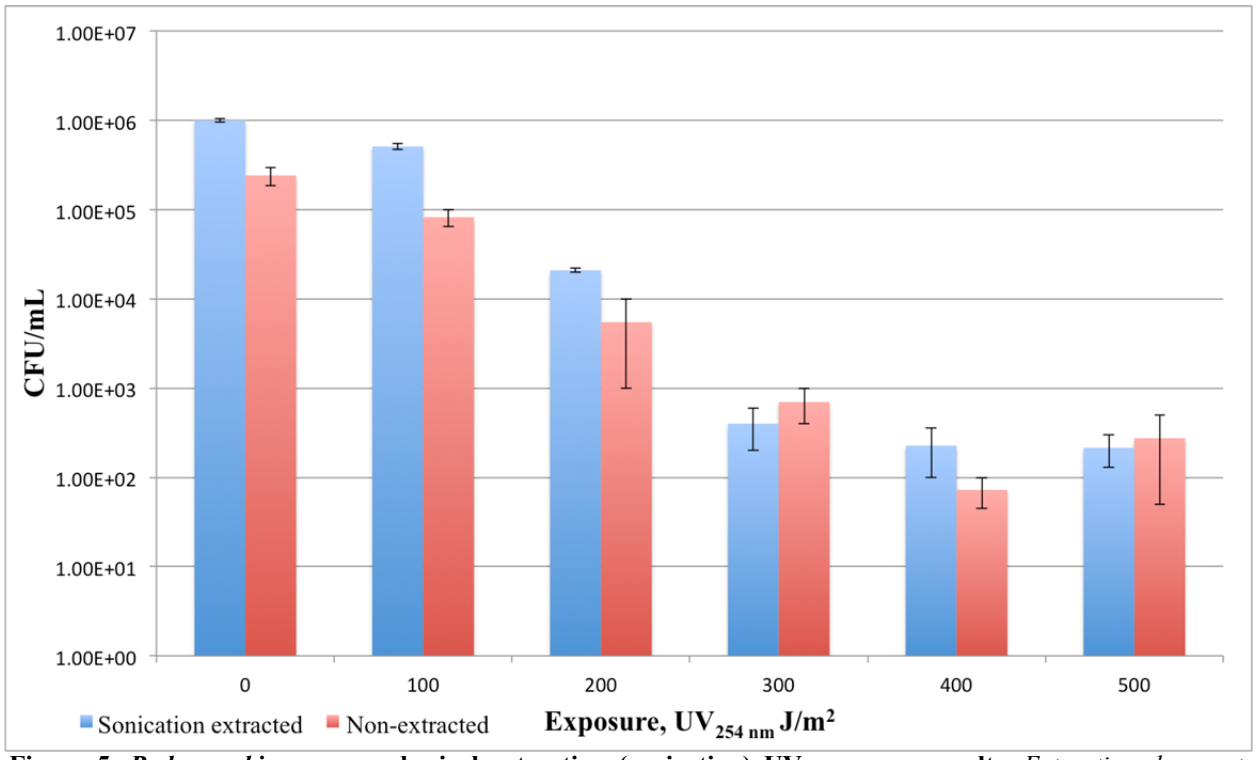

Figure 5. *B. horneckiae* spores physical extraction (sonication) UV exposure results. *Extraction does not significantly impact resistance to UV exposure when compared with non-extracted spores.*

D. UV Exposure Tests

Figure 6 demonstrates that *B. horneckiae* spores exposed to the full UV spectrum from 200-400 nm exhibited similar decreases in viability with increasing exposure as was shown in the extraction UV experiments. In addition, the dark and control samples (both unexposed) demonstrate that the PVA peeling process recovers the majority of spores that were deposited on the coupon. The maximum exposure level that resulted in viable spores was 5.0×10^5 kJ/m^2; although, one caveat was that only one of the triplicate samples at that exposure level produced viable spores. The exposure level at 1.5×10^5 kJ/m^2 led to viable spores from all triplicate samples. In addition, all samples that produce 0 colonies when diluted also produced 0 colonies when placed in pour plates. However, all the coupons of these "0 CFU" samples produced viable cells in the liquid TSB medium. Conclusions cannot be made on the original number of viable spores from the TSB cultures since it would only take one or a few viable cells, located in areas such as one on the coupon's edge or even on the unexposed side, to produce a colony.

Although the environment, dry versus liquid, and initial concentration of spores (10^7 CFU) of these coupon samples were different than the ones used for the extraction UV experiments (10^6 CFU/mL), it is notable that both experiments produced roughly 10^2 CFU (or 10^2 CFU/mL) at their final exposure levels. For the coupon samples, the viability quickly drops and remains around 10^3 and 10^2 CFU for multiple exposures. It is not clear why this relative stability in CFU numbers occurs, but it may be an indication that a certain subpopulation of spores in some deeper area of their biofilm are shielded from the radiation.

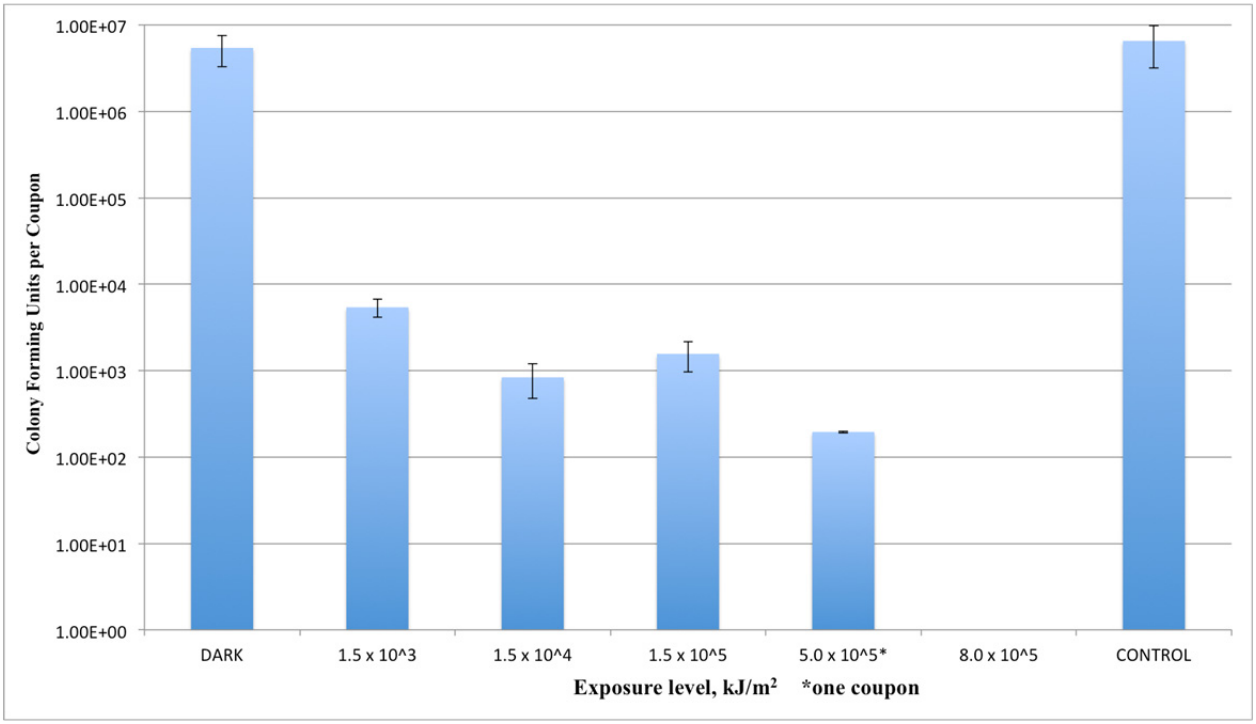

Figure 6. *B. horneckiae* coupon UV exposure results. *Samples were exposed to UV spectrum of 200-400 nm. DARK and CONTROL samples were unexposed. Each sample represents an average of three coupons (samples) except for 5 x 10^5 kJ/m^2 exposure.*

Acknowledgements

This research was carried out at the Jet Propulsion Laboratory, California Institute of Technology, and was sponsored by USRP and the National Aeronautics and Space Administration. Many thanks are given to Dr. Parag Vaishampayan and Dr. Kasthuri Venkateswaran for their guidance with this research and exposing me to other techniques like bioinformatics. Thanks are also given to Wayne Schubert for use of his sonicator and assistance in its setup and tuning. I thank Emilee Bargoma for the engaging discussions at my desk and letting me follow her molecular biology projects. I thank the other members of JPL's Biotechnology and Planetary Protection Group for their input and discussions, including those I got to know (J. Andy Spry, Robert Beaudet, Myron T. La Duc, Moogega Cooper, Fei Chen, and Ying Lin) and those I just met. I thank the NASA USRP program for funding me during this research.

References

[1]Newcombe, D. A., et al., "Survival of spacecraft-associated microorganisms under simulated Martian UV irradiation," *Applied Environmental Microbiology,* Vol. 71, No. 12, 2005, pp. 8147–8156.

[2]Ponce, A., Connon, S. A., and Yung, P. T., "Detection and Viability Assessment of Endospore-Forming Pathogens," *Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems,* edited by Zourob, M., Elwary, S., and Turner, A., Springer, New York, 2008, Chap. 19.

[3]Venkateswaran, K., "*Bacillus nealsonii* sp. nov., isolated from a spacecraft-assembly facility, whose spores are gamma-radiation resistant," *International Journal of Systematic and Evolutionary Microbiology*, Vol. 53, No. 1, 2003, pp. 165–172.

[4]Driks, A., "Overview: Development in bacteria: spore formation in *Bacillus subtilis*," *Cellular and Molecular Life Sciences* Vol. 59, No. 3, 2002, pp. 389–391.

[5]Kailas, L., et al., "Surface architecture of endospores of the *Bacillus cereus/anthracis/thuringiensis* family at the subnanometer scale," *Proceedings of the National Academy of Sciences U.S.A.,* Vol. 108, No. 38, 2011, pp. 16014-16019.

[6]Vaishampayan, P., et al., "*Bacillus horneckiae* sp. nov., isolated from a spacecraft-assembly clean room," *International Journal of Systematic and Evolutionary Microbiology*, Vol. 60, No. 5, 2010, pp. 1031–1037.

[7]Leighton, T. J., and Doi, R. H., "The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*," *Journal of Biological Chemistry,* Vol. 246, No. 10, 1971, pp. 3189–3195.

[8]Zhao, J., Krishna, V., Moudgil, B., and Koopman, B., "Evaluation of endospore purification methods applied to *Bacillus cereus*," *Separation and Purification Technology*, Vol. 61, No. 3, 2008, pp. 341–347.

[9]Thompson, B. M., Binkley, J. M., and Stewart, G. C., "Current physical and SDS extraction methods do not efficiently remove exosporium proteins from *Bacillus anthracis* spores," *Journal of Microbiological Methods,* Vol. 85, No. 2, 2011, pp. 143–148.